Characterization and polyanion-binding properties of purified recombinant prion protein

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Certain polysulphated polyanions have been shown to have prophylactic effects on the progression of transmissible spongiform encephalopathy disease, presumably because they bind to prion protein (PrP). Until now, the difficulty of obtaining large quantities of native PrP has precluded detailed studies of these interactions. We have over-expressed murine recombinant PrP (recPrP), lacking its glycophosphoinositol membrane anchor, in modified mammalian cells. Milligram quantities of secreted, soluble and partially glycosylated protein were purified under non-denaturing conditions and the identities of mature-length aglycosyl recPrP and two cleavage fragments were determined by electrospray MS. Binding was assessed by surface plasmon resonance techniques using both direct and competitive ligand-binding approaches. recPrP binding to immobilized polyanions was enhanced by divalent metal ions. Polyanion binding was strong and showed complex association and dissociation kinetics that were consistent with ligand-directed recPrP aggregation. The differences in the binding strengths of recPrP to pentosan polysulphate and to other sulphated polyanions were found to parallel their *in vivo* anti-scrapie and *in vitro* anti-scrapie-specific PrP formation potencies. When recPrP was immobilized by capture on metal-ion chelates it was found, contrary to expectation, that the addition of polyanions promoted the dissociation of the protein.

Key words: pentosan polysulphate, prion, prophylactic, scrapie, surface plasmon resonance.

INTRODUCTION

Transmissible spongiform encephalopathies (TSEs) are fatal neurodegenerative diseases that include scrapie of sheep, bovine spongiform encephalopathy and human Creutzfeldt–Jakob disease. During TSE disease progression, a host-encoded cell-surface glycoprotein, PrP^{C} (cellular prion protein), is altered to a partially proteinase K-resistant form, PrP^{Sc} (the scrapie-specific isoform of prion protein). This TSE-specific change is associated with an increase in the β-sheet structure of the protein and occurs without clearly identifiable covalent modifications to either the amino acid sequence or the C-terminal glycophosphoinositol (GPI) membrane anchor [1]. PrP^{Sc} accumulates in insoluble amorphous aggregates as scrapie-associated fibrils, which may be observed by electron microscopy [2], and, less commonly, as larger amyloid plaques, which may be visualized by light microscopy [3]. Although some TSE infectivity and PrP^{Sc} co-purify, the precise nature of the aetiological agent and a molecular understanding of TSE strain variation remain elusive [4].

Experimental animal models have demonstrated that, once clinical signs appear, TSEs are invariably fatal and that certain polysulphated compounds can delay disease if administered around the time of peripheral TSE inoculation [5]. Cell-culture studies have shown that these polyanions can decrease cell-surface PrP^{C} levels [6] and, with persistently scrapie-infected cells, reduce or permanently inhibit conversion of PrP^{C} to PrP^{Sc} [7,8]. With several polyanions this irreversible loss of PrP^{Sc} production is associated with complete loss of scrapie infectivity in subsequent animal titration experiments (C. Birkett, personal communication). Other studies have shown that PrP^{C} released from the surface of cultured neuroblastoma cells binds to heparin, a sulphated polyanion closely related to cellular glycosaminoglycans (GAGs), and that other polyanions can block this binding [8,9]. In addition, scrapie amyloid plaques have been shown to contain GAGs associated with PrP^{Sc} [3]. These data suggest that anti-scrapie polyanions act by competing directly with the binding of cellular GAGs to PrP^{C} and/or PrP^{Sc}. It is therefore important to study the binding of polyanions to PrP^{C} to provide information on the mode of action of these therapeutic compounds.

Only small amounts of native PrP^{C} can be extracted from brain, the most concentrated natural source [10–12], whereas many molecular structure and function analyses require larger amounts of purified, soluble and non-denatured protein. Several groups have expressed recombinant PrP (recPrP) in a range of gene-expression systems [13], but those capable of providing milligrams of recPrP per litre of culture have produced material that is denatured, truncated and/or fused to another protein. We describe the stable expression, in mammalian cells, of recPrP genetically modified to prevent GPI attachment. Milligram

Abbreviations used: DELFIA, dissociation-enhanced lanthanide fluorescence immunoassay; DMEM, Dulbecco's modified Eagle's medium; GAG, glycosaminoglycan; GMEM-S, Glasgow minimal essential medium for methionine sulphoximine selection; GPI, glycophosphoinositol; IDA, iminodiacetic acid; NTA, nitrilotriacetic acid; ORF, open reading frame; PNGase F, peptide N-glycosidase F; PrP, prion protein; *Prn-p*, prion protein gene; *Prp*, prion protein structural gene; PrP^{C}, cellular prion protein; PrP^{Sc}, scrapie-specific isoform of prion protein; PPS, pentosan polysulphate; recPrP, recombinant prion protein; SPR, surface plasmon resonance; TSE, transmissible spongiform encephalopathy.

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quantities of mature, full-length recPrP and two polypeptide fragments were purified by non-denaturing techniques and were characterized. The sulphated polyanion- and metal-ion-binding properties of the mixed-glycoform preparation of mature-length recPrP were assessed by surface plasmon resonance (SPR) techniques.

MATERIALS AND METHODS

All recPrP expression, purification, characterization and binding studies, except for studies with denatured protein, were performed in dedicated ACGM (Advisory Committee on Genetic Modification) level 3* biological-containment facilities [14].

Expression of recPrP

Genomic DNA from a VM-*Sinc*^{s7}/Dk mouse was used as the template for PCR amplification of part of the open reading frame (ORF) of the *Prn-p*A allele (where *Prn-p* is the prion protein gene) [15]. The PCR product was a *PrP* ORF (where *PrP* is the PrP structural gene) truncated by the insertion of a translation-stop codon immediately 3′ to the serine codon at residue 230. The DNA sequence encoding the messenger RNA immediately 5′ to the translation-initiation codon (underlined, see below) was replaced with a consensus sequence for high-level protein translation (i.e. 5′-…GCCGCCACCAUG…-3′) [16]. After DNA-sequence confirmation the mutated *PrP* ORF was subcloned into the mammalian expression vector pEE6HCMVne [17]. The resultant plasmid, pIAH4Aneo, was transfected into CHOL761h cells [18] and stable transfectants were selected using 1 mg/ml G418 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1 % (v/v) non-essential amino acids and 10 % (v/v) heat-inactivated foetal calf serum (Life Technologies) [19]. Stable transformants were cloned and screened for the production of secreted, truncated, recPrP by Western-blot analysis (see below). From these a cell line, DB11, was selected for recPrP production.

The truncated *PrP* ORF used in plasmid pIAH4Aneo was further mutated to prevent attachment of N-linked glycans to the protein. The DNA for the asparagine codons at PrP residues 180 and 196, the two glycosylation sites, was mutated to encode threonine at both residues (AAT mutated to ACC and AAC to ACC, respectively). In the course of this PCR-mediated mutagenesis an unplanned point mutation occurred at the position corresponding to PrP residue 99. This mutation substituted the codon AAC for AAT but, since both encode asparagine, this change was not corrected. This mutant *PrP* ORF was then ligated into the mammalian expression vector pEE14 [19] to give plasmid pIAH12. Stable transfectants of CHOL761h cells were selected in GMEM-S (Glasgow minimal essential medium for methionine sulphoximine selection [19]) that contained 10 % (v/v) heat-inactivated, dialysed, foetal calf serum using 25 μM methionine sulphoximine [19]. Stable transformants were cloned and, after Western-blot analysis (see below), cell line 12.4F was chosen for the production of aglycosyl recPrP.

Purification of recPrP

Ten roller bottles containing 80 % surface-confluent cultures of cell line DB11 were washed with PBS and incubated for 6 days in 100 ml of serum-free, selection-free, DMEM including 20 μM sodium butyrate [20] and 1 % (v/v) Nutridoma SR (Boehringer). Leupeptin and chymostatin were added on alternate days (final concentration, 10 μM). Culture medium containing secreted recPrP was separated from cell debris by centrifugation (10000 ***g***, 30 min) and buffered by the addition of 0.1 vol. of 0.5 M Hepes/NaOH, pH 7.0. PMSF was added (final concentration, 0.1 mM) and the medium was applied, at a flow rate of 6 ml/min, to a 10-ml S-Sepharose fast-flow cation-exchange chromatography column (Pharmacia) pre-equilibrated with 50 mM Hepes/NaOH and 150 mM sodium chloride, pH 7.0. After washing, bound proteins were eluted with a buffered linear NaCl gradient (150–650 mM) at a flow rate of 4 ml/min over 148 min. The eluent was monitored by absorbance at 280 nm and the salt gradient was monitored by conductivity measurement. Peak absorbance fractions were pooled (80 ml total) and applied, at 4 ml/min, to a 2-ml chelating Sepharose fast-flow chromatography column (Pharmacia) charged to 80 % binding capacity with zinc ions and equilibrated in 50 mM Hepes/NaOH/0.5 M NaCl, pH 7.4. After washing, bound proteins were eluted with a buffered linear imidazole gradient (0–100 mM) at 1 ml/min over 35 min. Genetically aglycosyl recPrP was purified using similar methods but by growing 12.4F cells in GMEM-S plus 10 % (v/v) dialysed foetal calf serum and then by accumulating recPrP in serum-free GMEM-S.

recPrP from cell line DB11 was also purified, with reduced metal-ion carry-over, by reversing the order of the chromatographic steps. Clarified recPrP-containing DMEM medium was buffered by the addition of 0.1 vol. of 0.5 M Hepes/1.5 M NaCl, pH 7.4, then mixed with zinc-charged buffer-equilibrated chelating Sepharose. After overnight incubation at 4 °C, the column was packed and the above imidazole gradient applied. Eluted fractions were diluted 4-fold, in 50 mM Hepes/NaOH, pH 7.0, prior to cation-exchange chromatography.

Samples for SDS/PAGE gels (12.5 %, w/v, acrylamide/bisacrylamide, 10 cm × 10 cm) were prepared by precipitation of protein solutions (usually 300 μl) with three vols. of acetone at −20 °C for 10 min followed by 10 min of centrifugation at 6500 ***g***. Precipitated proteins were resuspended in 30 μl of SDS/PAGE sample buffer containing 10 mM dithiothreitol and 10 % (v/v) 2-mercaptoethanol. Gels were loaded with 10 μl of sample per lane. All Western-blot analyses were carried out on Immobilon-P membranes (Millipore) blocked with non-fat milk and were probed with chicken polyclonal antiserum 1659 [21]. Immunoreactive proteins were detected using rabbit polyclonal anti-chicken IgY antiserum conjugated to horseradish peroxidase (Jackson ImmunoResearch) and were visualized by enhanced chemiluminesce (Amersham).

Bicinchoninic acid and Coomassie Plus protein-assay kits were used according to the manufacturer's instructions (Pierce), with BSA standards prepared in the appropriate basal growth media or elution buffer. Two protein assays were employed since no single assay was found that was tolerant of both the growth medium and imidazole in the metal-chelate chromatography eluent. Estimates of recPrP concentration were made by a dissociation-enhanced lanthanide fluorescence immunoassay (DELFIA, Wallac) [22]. recPrP was captured on microtitre plates passively coated with the anti-recPrP mouse monoclonal antibody, FH11 (a gift from C. Birkett, Institute for Animal Health, Compton, Berks., U.K.), and was detected using an anti-recPrP, *c-myc* epitope-tagged, synthetic, bacterial phage display monoclonal antibody (a gift from L. Davies and J. Young, Institute for Animal Health, Compton, Berks., U.K.). Bound phage antibody was detected by a europium chelate-labelled, anti-*myc*, mouse monoclonal antibody (a gift from G. Barnard, Institute for Animal Health, Compton, Berks., U.K.). After washing off unbound reagent, the europium was dissociated from its chelate, re-chelated, and the fluorescence was measured in a time-resolved fluorimeter [22].

Purified recPrP (0.1–10 μM) was stored at 4 °C in the metal-

chelate chromatography eluent buffer. Solubility was lower in neutral pH buffers containing less than 0.5 M salt and was decreased with increasing protein concentration or freeze–thaw treatment. These losses could be reduced by the addition of micellar concentrations of non-denaturing detergents.

Characterization of recPrP

The presence of N-linked glycosylation was analysed by digestion of detergent-denatured purified recPrP proteins with peptide N-glycosidase F (PNGase F) as recommended by the manufacturer (New England Biolabs). Proteinase K digestions were performed on 9.5 μM recPrP in metal-chelate chromatography eluate buffer using 50 μg/ml proteinase K at 37 °C for 1 h. After digestion, PMSF was added to a final concentration of 1 mM and the reaction cooled on ice for 30 min. Digested samples were acetone precipitated and subjected to Western-blot analysis (as above).

Online HPLC-electrospray MS analyses were performed by use of a Quattro II tandem quadrupole instrument (Micromass). Purified recPrP samples were desalted with a protein-trap cartridge (Michrom BioResources) by washing with water and 0.1 % (v/v) trifluoroacetic acid. Bound proteins were eluted on to a C4 reverse-phase chromatography column (220 mm × 2.1 mm, 7 μm bead size, 300 Å pore size; Perkin Elmer) and separated using a gradient of 30–70 % acetronitrile and 0.1 % (v/v) trifluoroacetic acid at 200 μl/min (140C Microgradient pump, Applied Biosystems). Approx. 5 % of the eluent was directed, via a UV absorbance detector, to the source of the mass spectrometer. The capillary voltage was held at 3.25 kV, whereas the voltage applied to the sampling cone was ramped as the instrument was scanned over from a mass-to-charge ratio (m/z) of 500–2000.

SPR

SPR binding studies were performed at room temperature with a BIALite molecular-interaction biosensor instrument (Biacore AB). SPR biosensor chips were purchased from the manufacturer and derivatized as required. Porcine intestinal mucosal heparin (Sigma H 3393) was N-acetylated using acetic anhydride. N-acetylated heparin and pentosan polysulphate (PPS, Sigma) were both coupled to putrescine at their reducing ends by reductive amination using a borane–pyridine complex [23] and biotinylated using excess biotin sulpho-*N*-hydroxysuccinimide ester. Biotinylated polyanions were immobilized by capture on either SA or SA5 streptavidin-coated biosensor chips. Except where stated, the binding of soluble recPrP to immobilized polyanions was studied in 10 mM Hepes/NaOH/150 mM NaCl, pH 7.4, containing 0.2 % (w/v) Zwittergent 3–12 detergent (Calbiochem). After each experiment, the biosensor chip was regenerated using 10 μl of 1 M NaCl and then 5 μl of 12.5 mM NaOH. Competitive-inhibition SPR experiments were performed in the above binding buffer but with a different detergent, 1.2 % (w/v) nonyldimethylamine oxide (Fluka), by pre-incubating recPrP for 30 min at room temperature with a 500 μg/ml solution of a competing polyanion prior to the measurement of binding to immobilized PPS. The free polyanions used were PPS, dermatan sulphate (chondroitin sulphate B, Sigma C 2413) and N-acetylated heparin.

Nitrilotriacetic acid (NTA) nickel-ion-chelation biosensor chips were charged with 500 μM nickel(II) chloride in 300 mM Tris/HCl, pH 7.5, prior to equilibration in recPrP-binding buffer. After recPrP binding, the effects of the addition of heparin in recPrP-binding buffer were monitored.

The iminodiacetic acid (IDA) metal-ion-chelation biosensor chips were constructed by synthesizing aminohydroxypropyl IDA by the alkaline coupling of epibromohydrin and IDA followed by treatment of the product with aqueous ammonia. The resultant chelating compound was linked covalently to commercial CM5 carboxymethyl dextran chips using a modification of the manufacturer's amine-coupling procedure (90 μl of *N*-hydroxysuccinimide/ethyl dimethylaminopropyl carbodiimide solution then 90 μl of chelate at 10 mg/ml in 20 mM sodium bicarbonate buffer, pH 8.2/1 M NaCl, flow rate 3 μl/min, followed by excess ethanolamine to block unreacted surface esters). IDA biosensor chips were charged with copper ions using 500 μM copper(II) chloride in 300 mM Tris/HCl, pH 7.5, followed by equilibration in recPrP-binding buffer.

After use, both types of chelation chip were regenerated by removal of bound metal ions using 50 mM Hepes/NaOH/500 mM NaCl/300 mM EDTA, pH 7.4.

RESULTS AND DISCUSSION

Production and purification of recPrP

Murine recPrP, truncated at the position expected for GPI-anchor attachment, accumulated in the DB11 cell-culture medium and was purified using low-pressure cation-exchange (Figure 1) and immobilized metal-chelate affinity chromatography

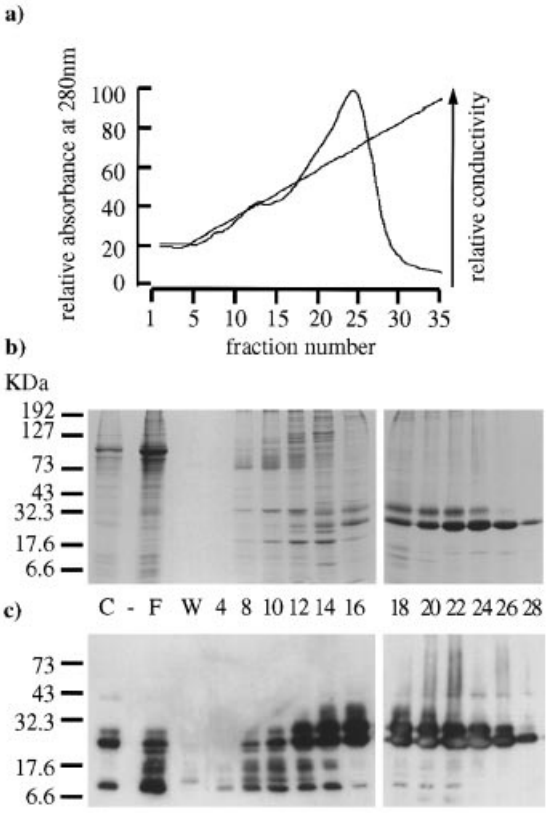

Figure 1 Purification of recPrP from cell line DB11 culture supernatant by cation-exchange chromatography

(**a**) Absorbance of protein-containing fractions (undulating line) eluted by an increasing concentration of NaCl (conductivity = diagonal line). (**b**) Coomassie Brilliant Blue stain image of SDS/PAGE gel (100-μl sample equivalent/lane). (**c**) Chemiluminescence image of Western-blot analysis of fractions taken before and throughout the elution (100-μl sample equivalent/lane; primary antibody, 1659). Lane C, DB11-cell-line-conditioned serum-free medium; lane F, flow-through material not bound to column; lane W, final column wash fraction. Other lane numbers correspond to the chromatography elution fraction numbers in both (**a**) and Table 1.

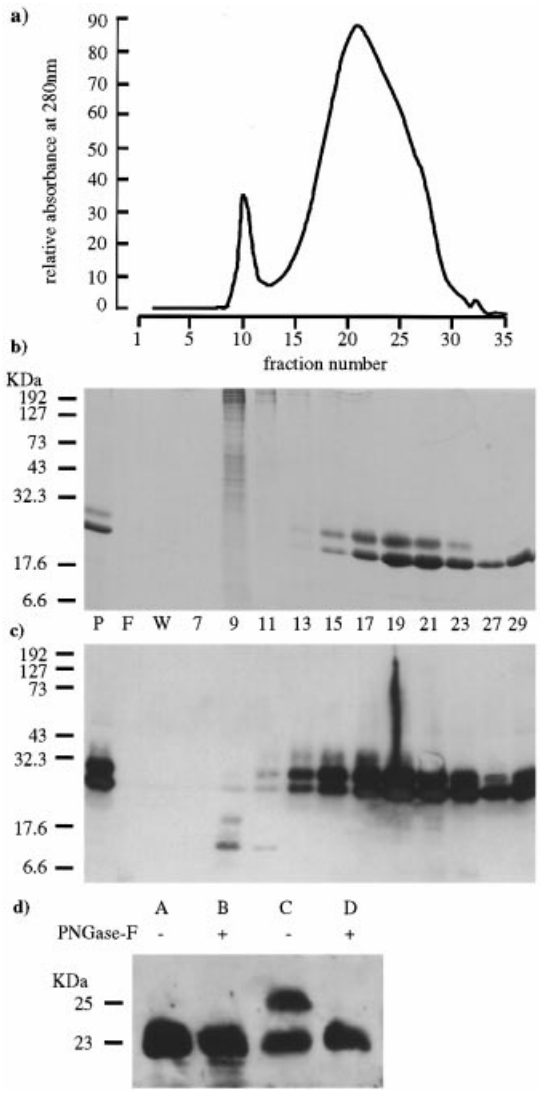

Figure 2 Further purification of recPrP-containing fractions pooled after cation-exchange chromatography by zinc-chelate chromatography

(**a**) Absorbance of protein-containing fractions eluted by an increasing concentration of imidazole. (**b**) Coomassie Brilliant Blue stain image of SDS/PAGE gel (100-μl sample equivalent/lane). (**c**) Chemiluminescence image of Western-blot analysis of fractions taken before and throughout the elution (100-μl sample equivalent/lane; primary antibody, 1659). Lane P, cation-exchange peak pool (fractions 16–25, Figure 1); lane F, flow-through material not bound to column; lane W, final column wash fraction. Other lane numbers correspond to the fraction numbers in both (**a**) and Table 1. (**d**) Chemiluminescence image of Western-blot analysis of purified recPrP after PNGase F digestion (100-μl equivalent/lane; primary antibody, 1659). Lane A, purified, genetically aglycosyl recPrP (residues 23–230); lane B, material in lane A digested with PNGase F; lane C, purified DB11 recPrP (pooled fractions 15–25 inclusive, see **a**); lane D, material in lane C digested with PNGase F.

steps (Figures 2a–2c). (Note: Chromatography fraction numbers also correspond to gel lane numbers.) The purification was assessed by Coomassie Brilliant Blue-stained SDS/PAGE gels (Figures 1b and 2b), Western-blot analyses (Figures 1c and 2c), DELFIA immunoassay (Table 1) and by two colorimetric protein assays (Table 1). In the DELFIA assay, the two monoclonal antibodies used for recPrP capture and subsequent detection recognized epitopes in the N-terminal half of the protein. As a consequence, both mature-length recPrP and any N-terminal fragments of the protein were measured together.

Three major immunoreactive recPrP species were observed in the DB11-conditioned medium, of approx. 9, 23 and 25 kDa (Figure 1c, lane C). Most of the non-PrP material and some of recPrP material did not bind to the column (Figures 1b and 1c, lanes F). The bound 23- and 25-kDa proteins, together with small amounts of a previously undetected 27-kDa recPrP species, were eluted from the cation-exchange column between 300 and 500 mM NaCl together with small amounts of the 9-kDa form (Figures 1b and 1c, lanes 16–26). Non-PrP proteins eluted throughout the salt gradient (Figure 1b). Fractions 16–25 (Figure 1a) were pooled for further purification. A DELFIA value of 4.8 mg of recPrP in 6.9 mg (bicinchoninic acid) or 9.9 mg (Coomassie Plus) of total protein was obtained after this first step (Table 1). Purification yields were difficult to estimate as the DELFIA assay measured a value of only approx. 250 μg of recPrP prior to purification (Table 1) due to interference effects from the cell-culture media. Attempts to minimize these effects by diluting the medium caused an unacceptable loss to the signal-to-background-noise values.

The pooled cation-exchange fractions (see above; Figures 2b and 2c, lanes P) were further purified by immobilized zinc-chelate affinity chromatography (Figures 2a–2c). Little recPrP material was detected in the column flow-through (Figures 2b and 2c, lanes F), indicating that virtually all of the pooled protein was bound. Most of the non-recPrP material that was also bound, together with a lower-abundance 14-kDa recPrP fragment, was eluted early in the elution gradient (at $\leqslant$ 40 mM imidazole; Figures 2b and 2c, lanes 9–11). recPrP forms of approximate masses 23, 25 and 27 kDa eluted later (around 85 mM imidazole) and these, together with small amounts of a co-purifying 9-kDa recPrP species, were pooled (Figure 2a, fractions 15–25). The final yield of purified mature-length recPrP and N-terminal fragments was 3.6 mg at a concentration of 225 μg/ml (Table 1) and was judged to be 98 % recPrP by silver-stained SDS/PAGE analysis of serial dilutions (results not shown).

Yields and purity of aglycosyl recPrP obtained from cell line 12.4F cultures were close to those obtained with cell line DB11, despite the differences in the cell growth and selection media.

Our data demonstrate that, despite lacking the GPI moiety, the purification properties of our murine recPrP were broadly similar to those of wild-type PrP^c purified from hamster brain tissue [10–12].

Our production process, using cell lines DB11 and 12.4F, represents a considerable improvement in efforts to obtain the quantities of mature-length recPrP necessary for structural and functional analyses, as it is the first reported non-denaturing purification of milligram quantities of mature-length recPrP.

Our cell line 12.4F is similar to some of those made by Blochberger et al. [13], as they used the same gene-expression vector, pEE14 [19]. They differ in that Blochberger et al. [13] expressed both wild-type and truncated Syrian hamster *PrP* ORFs, not the mouse equivalents. They also used Chinese hamster ovary K1 cells as their expression host, whereas we used CHOL761h cells [19]. CHOL761h cells are Chinese hamster ovary K1-based but also express a mutant form of the adenovirus E1A gene product at a level optimized for maximal stimulation of the vector's recPrP-expression control region. It is not clear if truncated hamster recPrP was made at levels similar to those in our truncated mouse recPrP cell lines, because Blochberger et al. [13] concentrated their quantification analyses on recPrP with an intact membrane-anchor signal. In order to increase yields of recPrP they attempted amplification of the expression-vector copy number in their cell lines [19]. Cell lines with increased resistance to drug selection were obtained but it is not clear if they exhibited increased copy number or elevated recPrP-ex-

Table 1 Summary of recPrP purification steps

N.D., not determined.

Sample	Volume (ml)	Spectrophotometric assays: total protein (mg)		DELFIA: total mature-length recPrP and N-terminal fragments (mg)	Purity (%)
		BCA	Coomassie Plus		
Media from cells	1000	N.D.	293	0.25*	–
Cation-exchange peak pool	90	6.9	9.9	4.8	48–70
Metal-chelate peak pool	16	3.6	N.D.	3.6	100†

* This result was too low due to interfering effects of media constituents. See text for discussion.
† 98% pure on silver-stained polyacrylamide gel.

pression levels. We attempted to increase recPrP yields from cell line 12.4F and our other pIAH12-based cell lines by similar procedures. Despite phenotypic increases in drug-resistance levels, we were unable to generate cell lines that gave increased productivity. This finding may not be specific to recPrP, as in other reports the stimulatory effects of the adenovirus E1A background have not been additive, with the stimulation founded on increased gene copy number [18].

Characterization of purified recPrP

Purified recPrP material (Figure 2, pooled fractions 15–25, and Table 1) was used for N-linked glycosylation analyses. Western-blot analysis of recPrP following PNGase F digestion showed that the 25-kDa recPrP form was no longer present, whereas the 23-kDa form was unaltered (Figure 2d). This is consistent with the removal of a single glycosylation chain from the glycosylated recPrP but does not identify whether each of the N-glycosylation sites was occupied independently. Confirmation that the 25-kDa form was glycosylated recPrP and that the 23-kDa form was non-glycosylated was obtained by simultaneous digestion of genetically aglycosyl recPrP that had been purified from cell line 12.4F. The digested genetically aglycosyl recPrP was not reduced in size (Figure 2d).

The majority of the mature-length mouse recPrP produced was non-glycosylated, consistent with the underglycosylation of C-terminally truncated hamster recPrP seen by both Kocisko et al. [24] and Blochberger et al. [13]. Nevertheless, small amounts of a 27-kDa recPrP form, a mass consistent with diglycosylated protein, can be seen by Western-blot analysis of heavily loaded SDS/PAGE gels (Figures 1c and 2c). The 23-, 25- and 27-kDa recPrP forms were fully sensitive to proteinase K digestion (results not shown), indicating that they did not have the proteinase K resistance associated with PrP^{Sc} [1].

The electrospray mass spectrum of purified recPrP (Figure 2, pooled fractions 15–25) is shown in Figure 3. This spectrum shows two series of peaks, labelled A and B. Series A corresponds to a substance of mass 22934.2 ± 4.5 Da (Figure 3, inset). This is within 4 Da of the theoretical mass of the murine *Prn-p*A gene product from the lysine at residue 23 (the first amino acid in the mature-length protein) to the serine at residue 230 (the last amino acid expected at the end of the genetically truncated recombinant protein) [15]. The protein peaks are not resolved fully and include a substantial higher-mass tail, presumably due to the adduction of sodium ions. The identity of the protein was confirmed by mass-spectral analysis of peptides resulting from a chymotryptic digest (results not shown). The hamster recPrP mammalian-cell expression studies of Rogers et al. [25], Kocisko et al. [24] and Blochberger et al. [13] showed that C-terminally truncated rodent recPrP is secreted and is matured by cleavage of the N-terminal nascent signal peptide. Our data provide the first high-resolution confirmation that maturation cleavage of secreted recPrP is identical to that found with PrP^{C} *in vivo* [12].

The B series of peaks in the mass spectrum leads to a mass of 23278.3 ± 2.0 Da (Figure 3, insert). This mass is 344 Da higher than the measured mass of mature-length recPrP and co-elutes from the reversed-phase column, suggesting the presence of either a post-translational modification or a substance adducted to recPrP. Studies to identify this mass are in progress.

Masses corresponding to glycosylated, mature-length recPrP forms were not obtained. Only small quantities of glycosylated recPrP were present relative to the aglycosyl recPrP and, hence, it is probable that insufficient quantities and/or glycosylation heterogeneity precluded measurement of these species. Their presence may, however, have contributed to the chemical noise level observed (Figure 3).

A recPrP polypeptide of approx. 9 kDa was present at a low level in the pool of purified mature-length recPrP material (Figure 2, fractions 15–25). A mass of 9008 ± 0.3 Da was measured by MS, in good agreement with the theoretical mass of a murine PrP fragment from the mature N-terminus at lysine amino acid residue 23 to the lysine residue at position 109 inclusively (results not shown). The 9-kDa form contains many basic amino acid residues and, hence, this explains the co-purification of this fragment with mature-length recPrP by cation-exchange chromatography, since both have highly basic pI values. In addition, because both of these forms contained the five octa-repeat metal-binding domains [26–28], they also co-purified by metal-chelate chromatography. Semi-purified 14-kDa recPrP material was obtained by separate zinc-chelate chromatography of the cation-exchange purification fractions 6–15 inclusive (Figure 1) The material eluted from the column in 30 mM imidazole (results not shown). Mass-spectrometric analyses identified a molecule of mass 13940 ± 1.1 Da, in close agreement with the mass of a C-terminal fragment of recPrP extending from a histidine at amino acid residue 110 to the end of the mature amino acid sequence (results not shown). A higher-mass species, as found with mature-length recPrP, was also observed in some of these analyses. This C-terminal fragment contains both sites for N-linked addition of sugar chains but, again, no glycosylated forms were observed in any of the mass analyses (results not shown).

The identification of the 9- and 14-kDa fragments shows that some of the mature-length 23-kDa recPrP was cleaved between residues lysine 109 and histidine 110. Our 14-kDa murine recPrP fragment is consistent with the expected size for an aglycosyl form of the PrP^{C}-II species that is formed by proteolytic cleavage of mature-length PrP^{C}-I material purified from hamster brains

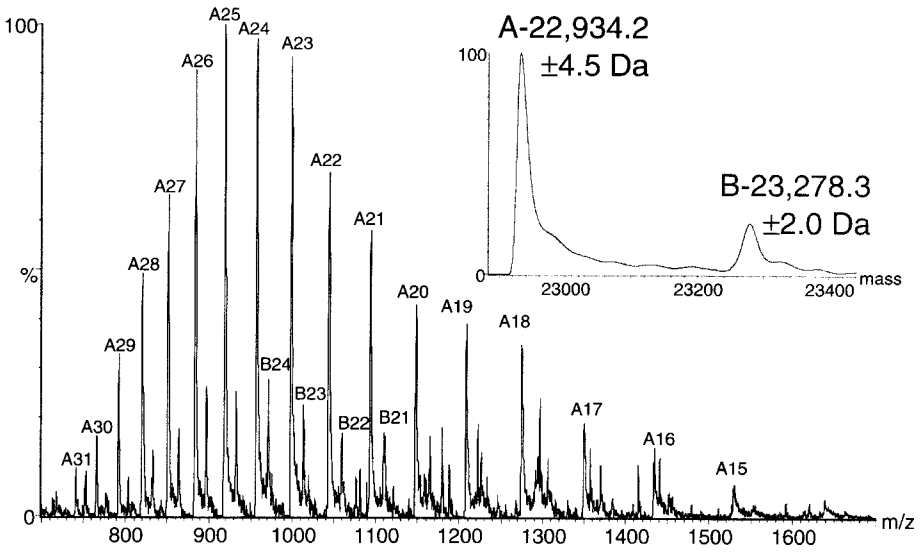

Figure 3 Electrospray mass spectrum of mature-length recPrP

Inset: deconvoluted mass spectrum. For details of series A and B, see text.

both *in vivo* and *in vitro* [10,12]. Our findings are the first precise identification of the internal cleavage site of a rodent PrP. Evidence for the homologous cleavage of human brain PrP^C *in vivo* has come from Chen et al. [29]. In addition, recombinant chicken PrP expressed in rodent cells is cleaved at a peptide bond that is adjacent to this cleavage site [30]. These data support the view that, at least with respect to its accessibility to internal proteolytic cleavage, our recPrP has a structure that is similar to native PrP^C.

SPR binding studies

The BIALite molecular-interaction biosensor instrument allows continuous SPR measurement of the binding of soluble macromolecules to ligands immobilized on a biosensor chip. The measurements are displayed as a graphical 'sensorgram' and the kinetics of binding are assessed by analysis of these binding curves. Polyanion-binding studies were performed using the purified mixture of mature-length recPrP glycoforms (Figure 2, pooled fractions 15–25 and Table 1).

recPrP binding to immobilized polyanions

PPS is a potent anti-scrapie compound [5] and is chemically more homogeneous than N-acetylated heparin and, therefore, was thought to be more likely to produce good kinetic binding data, whereas N-acetylated heparin is more closely related to cellular GAGs. Both of these ligands were biotinylated by derivatization of a putrescine linker attached to their terminal reducing sugar and then captured on streptavidin-coated SPR chips. The streptavidin capture was also expected to reduce any detrimental effects on binding studies caused by electrostatic repulsion between the polyanions and the carboxymethyl dextran matrix of the chip. In our attempts to obtain good data, a range of surface concentrations of both of these ligands were studied.

Differing amounts of recPrP were applied to these biosensor chips in successive experiments. The association phase of the binding was monitored for 660 s and the dissociation phase was followed after the protein solution was replaced with binding buffer only (Figure 4). Poor binding was observed in the Hepes-buffered-saline solution, recommended by the manufacturers of the SPR instrument, especially at low recPrP concentrations. Good binding was observed at physiological pH and ionic strength only when EDTA was omitted from the binding buffer or was present at sub-millimolar levels. The binding was found to be reproducible in physiological buffers only if micellar levels of a non-denaturing detergent were present, to reduce aggregation, precipitation and/or non-specific absorption of the protein. In control experiments, recPrP did not bind to unmodified streptavidin chips. Likewise, no binding to immobilized polyanions was seen with control material purified from conditioned medium from the non-transformed host-cell line, CHOL761h. This indicates that binding was due solely to recPrP and could not be ascribed to the approx. 2% of non-recPrP material present in the purified fractions used.

The binding between recPrP and both immobilized polyanions showed complex association and dissociation kinetics (Figure 4)

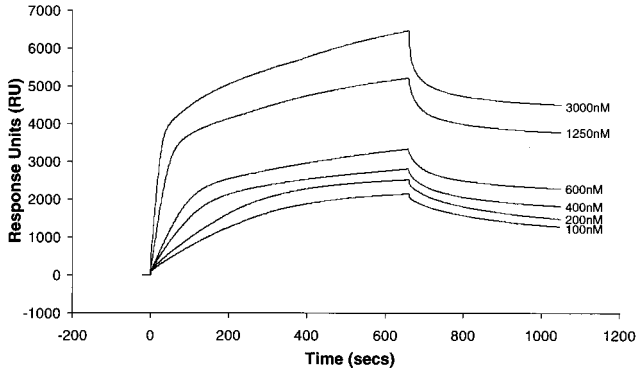

Figure 4 SPR sensorgrams of purified recPrP binding to immobilized PPS

A range of recPrP concentrations was injected over an immobilized PPS chip. Association phase, 0–660 s; dissociation phase, 660 s onwards (470 response units, RU, of PPS immobilized, recPrP injected at 5 μl/min).

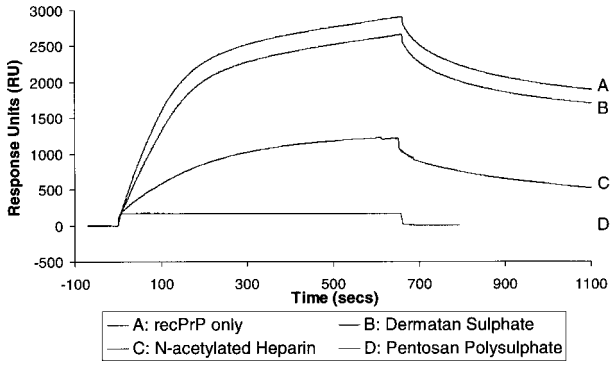

Figure 5 SPR sensorgrams of the inhibition by three sulphated polyanions of the binding of recPrP to immobilized PPS

Sensorgrams of recPrP binding: curve A, without inhibitor; curve B, after pre-incubation with soluble dermatan sulphate; curve C, after pre-incubation with soluble heparin; and curve D, after pre-incubation with soluble PPS. Association phase, 0–660 s; dissociation phase, 660 s onwards (470 relative units, RU, of PPS immobilized, 300 nM recPrP with or without 500 μg/ml competitor polyanion injected at 5 μl/min).

Table 2 Summary of the effects on immobilized PPS binding caused by various treatments to the metal-ion status of recPrP

IonX/IDA, recPrP material purified by the standard methods; IDA/IonX, recPrP material purified with the chromatography steps reversed. N.D., not determined.

	Increase in SPR response (response units) after 240 s	
Additions to binding buffer	IonX/IDA purification	IDA/IonX purification
None	400	100
3 mM EDTA	150	N.D.
1 mM $ZnCl_2$	N.D.	150

that precluded measurement of either the association or dissociation rate constants even when attempts were made to fit these data to models of complex binding interactions. The range of concentrations of both the polyanions and recPrP studied indicated that mass-transport limitation and/or ligand re-binding effects were not causative factors for this complexity. It was also unlikely to have been due to the mixture of recPrP glycoforms used, despite evidence from other systems (e.g. recombinant antithrombin III expressed in hamster cells, where N-glycosylation reduces heparin binding [31]), as similar binding kinetics were observed with aglycosyl recPrP purified from cell line 12.4F (results not shown). The complexity of the kinetics, therefore, is not thought to be artifactual, polyanion-specific or the product of different ligand-binding affinities of the constituent glycoforms. An explanation for the complexity of the kinetics is that the polyanions function as polyvalent ligands participating in a range of different interactions that are measured simultaneously.

When complex kinetics are observed it is often not possible to obtain binding-rate constants using simple curve-fitting equations and from these to determine binding affinity. Nevertheless, under normal circumstances, the equilibrium binding affinity (and the stoichiometry of the binding) can be estimated from the steady-state equilibrium values obtained over a range of concentrations of the two binding partners [32]. In this study, the recPrP–polyanion binding responses never achieved steady-state binding equilibria (i.e. plateaux on the SPR sensorgrams) because the response signal continued to increase as long as excess recPrP was present. This is consistent with ligand-directed recPrP self-aggregation and contrasts with results obtained with the binding of soluble histidine-proline-rich glycoprotein to immobilized polyanions where, despite the polyvalency of the ligands, steady-state equilibria where achieved [33]. The data presented here, therefore, lend support to the model proposed by Shyng et al. [6] that prophylactic polyanions cause aggregation of PrP^{c} at the cell surface and thereby block PrP^{Sc} formation by enhancing endocytosis of the cellular form.

The qualitatively rapid association and slow dissociation rates observed in Figure 4 suggest a high binding affinity between recPrP and PPS. This view is supported by the relatively harsh regeneration conditions required to remove the bound protein from the chip. Similar binding was observed with the N-acetylated heparin chip but with apparently slower association and faster dissociation rates, and milder regeneration conditions, suggesting that binding was weaker (results not shown).

Competitive SPR studies were performed in order to assess the relative binding affinities of three polyanions. recPrP was mixed with individual competitor polyanions (non-biotinylated) before injection over immobilized PPS. The sensorgrams in Figure 5 indicate that free PPS competes effectively with immobilized PPS (approx. 90% inhibition after 660 s), that free N-acetylated heparin has an intermediate effect (approx. 60% inhibition after 660 s) and that free dermatan sulphate has little effect (approx. 10% inhibition after 660 s). Although quantitative binding strengths cannot be obtained from these experiments, it can be concluded that the ligands possess binding strengths in the order: PPS > heparin > dermatan sulphate. This hierarchy parallels the abilities of these polyanions to both delay scrapie disease *in vivo* and block PrP^{Sc} formation in persistently scrapie-infected cells [7]. It also correlates with their ability to reduce cell-surface PrP^{c} levels [8], to block the binding of recPrP fusion proteins to cultured cells [6] and to act as competitors for the binding of PrP^{c} to heparin agarose [9]. The results presented here, therefore, provide the first evidence that the degree of TSE prophylaxis is related to the strength of binding of polyanions to PrP.

Effect of metal ions on polyanion binding

To reduce the carry-over of zinc ions leaching from the metal-chelate chromatography matrix during the purification procedure, recPrP was also purified by reversing the two chromatography steps. This gave material of comparable purity but of a lower final concentration. The results of subsequent SPR experiments are summarized in Table 2. These data show that if zinc-ion carry-over is diminished, PPS binding is stimulated by the re-addition of 1 mM zinc chloride (Table 2). This is consistent with the above observation that EDTA levels in the binding buffer must be reduced to allow full binding of recPrP to polyanions. These data provide the first clear evidence for an involvement of divalent metal ions in PrP–polyanion binding and link the observation by Shyng et al. [6] that polyanions enhance PrP^{c} endocytosis with similar effects of copper ions on the trafficking of the protein, reported by Pauly and Harris [34].

SPR has been used previously to demonstrate zinc-ion stimulation of heparin binding both to immobilized Alzheimer's disease amyloid precursor protein [35,36] and to histidine-proline-rich glycoprotein [33]. In the latter study, heparin binding was also promoted by low concentrations of copper(II) ions. Since copper(II) ions appear to be natural ligands for PrP^{c} [37,38] it is possible that they would have a greater effect on the polyanion

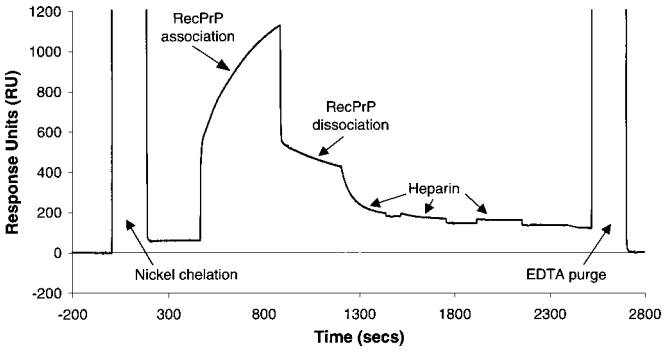

Figure 6 SPR sensorgram of purified recPrP binding to a NTA chip

Sequence of events: addition of nickel(II) chloride solution, which causes a large relative-unit (RU) response due to the bulk effects of the buffer. Return to recPrP-binding buffer (about a 60 RU increase in baseline due to nickel-ion chelation). Application of 1 μM recPrP, immediate bulk RU increase due to the buffer followed by recPrP binding. Return to binding buffer, immediate RU decrease due to the buffer followed by slow dissociation of recPrP from the chelate. Additions of free heparin at 1 mg/ml, increased the rate of recPrP dissociation seen, no RU increase even with a second or third addition of heparin. Regeneration of the NTA chip by removal of chelated nickel ions with EDTA, followed by a return to the original sensorgram baseline (flow rate 5 μl/min throughout).

binding of recPrP than do zinc ions. However, it was not possible to use our SPR-immobilized polyanion chips to evaluate the effects of copper ions on the binding of recPrP. Copper(II) ions bound to the carboxymethyl dextran matrix of the biosensor chip [39], and this permitted the binding of recPrP directly to the chip surface in the absence of the polyanions.

To investigate the binding of recPrP to immobilized metal ions, nickel-chelating NTA biosensor chips were used and a metal-ion-chelation chip, employing an IDA-chelation group, was constructed. The IDA-chelation functional group was the same as that attached to the chromatography matrix that was used in the metal-chelate purification step and it was capable of productively binding a wider range of divalent metal ions than the NTA group. Chelation of both nickel ions to the NTA chip and copper(II) ions to the IDA chip were stable (Figure 6) but, as with the zinc-chelate protein-purification matrix, when zinc ions were added to the IDA chip a significant leaching of metal ions from the chelate was observed. This loss of zinc ions precluded the study of the recPrP–zinc-ion interaction by SPR. In control experiments, the binding of recPrP to the NTA and IDA chips was found to be specific to the presence of nickel(II) and copper(II) ions respectively.

Figure 6 shows that recPrP associated rapidly with nickel ions captured on an NTA chip (steep upward curve) and dissociated slowly (shallow downward curve), indicative of a strong binding affinity of recPrP for nickel ions. These findings are in accordance with results of Stockel et al. [38] and Pan et al. [10], who demonstrated that, whereas copper(II) ions appear to be natural ligands for PrP^C, the protein will also bind nickel(II) ions with high affinity. Attempts were made to quantify the strength of binding of recPrP to both metal-ion chelates, but despite evidence for binding equilibria, the extent of binding could not be measured accurately, as regeneration of both types of chip was not reproducible.

Immobilized recPrP binding to polyanions

An SPR configuration with recPrP immobilized on the chip and with polyanions in solution avoids the potential for polyanions to act as polyvalent ligands and blocks any ligand-induced aggregation of the protein. More interpretable kinetic data would, therefore, be expected.

Several methods to immobilize recPrP on to SPR chips produced only partial successes but, nevertheless, generated interesting results. Direct coupling of recPrP to the carboxymethyl dextran chip via surface amines on the protein was successful but the immobilized recPrP did not bind free polyanions. Since lysine residues are often required for polyanion binding [40], a likely explanation for this is that coupling of the protein via these residues destroys binding. Indirect coupling of recPrP by either polyclonal antibodies or lectin immobilized on to the chip was successful, but no polyanion binding was seen. Again, it is likely that binding of recPrP to antibodies or lectins either masks or disrupts the site of polyanion binding or disrupts the conformation of the protein.

The immobilization of recPrP on to chelated metal ions suggested an alternative, potentially benign, method for studying the kinetics of recPrP association with free polyanions. However, the addition of heparin to recPrP bound to the nickel-chelated NTA chip did not result in the expected increase in SPR signal but, instead, heparin reduced the SPR response in a way consistent with enhanced dissociation of recPrP from the chip (Figure 6). A similar dissociation of protein was seen on the addition of either heparin or PPS to immobilized recPrP bound to the copper-chelated IDA chip, indicating that these phenomena are not metal-ion-specific, polyanion-specific or chelate-specific. The sensorgrams showed that the addition of polyanions reduced the SPR response to near its pre-recPrP levels but not to initial levels, indicating that metal ion was still bound to the chelate. These results show that the polyanions did not release recPrP from the chip by removing the metal ions from the chelates, but acted on the protein to cause it to dissociate from the immobilized metal ion. Application of fresh recPrP to the metal-chelate chip demonstrated not only that the protein was still able to bind efficiently to the chip surface but also that it restored the SPR response to a level similar to that found before the addition of heparin. This confirms that heparin removed the bound recPrP by interacting with the protein and did not compete with the protein to sequester the metal ions. This suggests that, although divalent metal ions appear to be required for binding of sulphated polyanions by PrP, the binding of polyanions causes the dissociation of the metal ions from the protein. These findings, therefore, provide further clues to the normal molecular function of PrP^C, where it presumably binds both divalent metal ions and cellular GAGs.

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